

Yield and Viability of Human Limbal Stem Cells From Fresh and Stored Tissue

Sharon L. Mason,¹ Rosalind M. K. Stewart,^{1,2} Carl M. Sheridan,¹ Fatemeh Keshtkar,¹ Paul Rooney,³ Eric Austin,³ Ursula Schlötzer-Schrehardt,⁴ Friedrich E. Kruse,⁴ and Stephen B. Kaye^{1,2}

¹Department of Eye and Vision Science, University of Liverpool, Liverpool, United Kingdom

²St. Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, United Kingdom

³Tissue Services, National Health Service Blood and Transplant, Speke, United Kingdom

⁴Department of Ophthalmology, University of Erlangen-Nürnberg, Erlangen, Germany

Correspondence: Carl Sheridan, Department of Eye and Vision Science, University of Liverpool, Liverpool, UK; carlos@liv.ac.uk.

Submitted: February 16, 2016

Accepted: May 10, 2016

Citation: Mason SL, Stewart RMK, Sheridan CM, et al. Yield and viability of human limbal stem cells from fresh and stored tissue. *Invest Ophthalmol Vis Sci.* 2016;57:3708–3713. DOI:10.1167/iovs.16-19354

PURPOSE. We compared cell number, putative stem cell markers, and clonogenic ability in fresh uncultured human limbal epithelial cells to that obtained from stored organ-cultured tissue.

METHODS. Cell suspensions were formed from fresh and organ culture-stored human limbal epithelium. Expression of putative stem cell markers Δ Np63 and TrkA was performed using immunofluorescent staining before culture. Colony-forming efficiency (CFE) assays were performed at first passage. The effects of tissue storage, age, and postmortem/culture times were analyzed in a general linear model.

RESULTS. Limbal tissue from 94 donors (34 fresh and 60 stored) was compared. Three times more cells were obtained per eye from fresh (35.34×10^4 ; SD, 17.39) than stored (11.24×10^4 ; SD, 11.57; $P < 0.01$) tissue. A higher proportion of cells from fresh tissue were viable (91.9%; SD, 5.7 vs. 85%; SD, 10.8) $P < 0.01$. Higher total cell expression of Δ Np63 (20.19×10^4 ; SD, 15.5 vs. 3.28×10^4 ; SD, 4.33) and TrkA (59.24×10^4 ; SD, 13.21 vs. 7.65×10^4 ; SD, 1.05) was observed in fresh than stored tissue ($P < 0.01$). Colony-forming efficiency was higher for fresh (1.42; SD, 0.12) than stored (0.43; SD, 0.15; $P < 0.01$) cells. For stored tissue only, there was a significant inverse relationship between donor age and total number of cells isolated ($R^2 = 0.27$, $P < 0.001$).

CONCLUSIONS. Storage of corneoscleral discs in organ culture medium leads to significant reduction in limbal epithelial cell number, expression of Δ Np63 and TrkA, and viability compared to fresh tissue. There is a smaller basal stem cell population in stored compared to fresh tissue.

Keywords: limbal epithelium, corneal organ culture, transplantation

In the healthy human eye, the corneal epithelium is renewed by stem cells located in the surrounding limbus. Limbal stem cell deficiency (LSCD) develops when these cells are diminished to such an extent that the corneal epithelium can no longer be maintained. This may result in persistent corneal epithelial defects, vascularization, and ingrowth of the conjunctival epithelium, with resultant pain and loss of vision.¹ Limbal stem cell deficiency may be managed by autologous or allogenic limbal stem cell transplantation, either directly, or following an ex vivo expansion cell culture step.^{2,3} This may help restore the corneal epithelium and reduce pain.⁴ In cases of bilateral stem cell deficiency, tissue is obtained from either a live or cadaveric donor.

It has been suggested that limbal tissue that is taken from a cadaveric donor should be placed onto the recipient's eye within 72 hours of the donor's death as a precaution against loss of cell viability with time.^{5,6} However, it is common practice in the United Kingdom and Europe to store corneoscleral discs intended for corneal transplantation for up to 3 weeks in an organ culture medium at 31°C.⁷ Other storage methods are common in other countries; for example, hypothermic storage at 4°C is used widely in the United States

and Asia. Due to the limited availability of fresh ocular tissue and the logistic advantages of stored tissue, corneoscleral rings have been used to generate limbal epithelial cultures for transplantation,^{8–11} usually as explant cultures. The methods used for storing corneas, however, have been designed to maintain endothelial cell health. Several studies have shown that the epithelium is harmed in organ culture and hypothermic storage media, resulting in a loss of cell layers^{12,13} and expression of proteins not usually found in the limbal epithelium.¹⁴ In addition, there is in vitro a reduced proliferative potential of limbal cells following storage in organ culture medium.^{15–18} However, the reasons for this decline have not been investigated. In particular, previous studies have focused on analyzing cell cultures from explants rather than from single cell suspensions, which has restricted the ability to examine cell number and viability at the time of culture. To investigate this process and the effect of storage on limbal epithelial cells, we examined cell numbers and viability as well as the expression of putative stem cell markers Δ Np63 and TrkA, and colony-forming efficiency (CFE) assays from fresh and stored limbal epithelium.



METHODS

Ethical approval was obtained and the research conformed to the tenets of the Declaration of Helsinki. Informed consent for the use of tissue was obtained from the next of kin of suitable donors. All reagents were from Sigma-Aldrich Company, Ltd, Gillingham, UK unless otherwise stated.

Limbal Tissue

Stored tissue was obtained from the Manchester Eye Bank, Manchester, UK and fresh tissue from the Liverpool Research Eye Bank, Liverpool, UK. For stored tissue, corneoscleral rims were obtained at the time of corneal transplantation from eyes that had been stored in organ culture medium (Dulbecco's modified Eagle's medium [DMEM] with Earle's salts, buffered with HEPES, and containing 26 mmol/L NaHCO₃, 2% fetal calf serum [FCS], 2 mmol/L L-glutamine, 1% penicillin-streptomycin, 1% fungizone) for up to 28 days at 37°C with 5% CO₂ before use. Limbal tissue was prepared by making incisions into the peripheral cornea and sclera, approximately 2 mm anterior and posterior to the corneoscleral limbus and excising a band of limbal tissue approximately 4 mm wide. Times from death to retrieval, retrieval to organ culture, and time in organ culture were recorded.

Digestion of Limbal Tissue and Viability Assay

Limbal tissue then was dissected into 16 even-sized pieces and exposed to 0.05% trypsin/0.02% EDTA for repeated 20-minute intervals at 37°C to dissociate the cells. After each digestion, the cell suspension was placed into limbal epithelial medium (3 parts low glucose [1 g/L] DMEM [Life Technologies, Paisley, UK] and 1 part Ham's F12 media [Life Technologies] containing 10% FCS, 1% penicillin-streptomycin, 1% fungizone, 5 µg/mL adenine, 5 µg/mL insulin, 1.4 ng/mL triiodothyronine, 12 µg/mL adenine, 0.4 µg/mL hydrocortisone, and 0.1 µg/mL epidermal growth factor). After the fourth cycle, the cells were pooled and resuspended in 0.5 mL of fresh limbal epithelial medium. Cells were counted and viability assessed using a 0.4% trypan blue assay and hemocytometer.

Immunocytochemical Staining for Limbal Stem Cell Markers

Immunocytochemical staining of newly isolated limbal cells was done for the putative stem cell markers ΔNp63 and TrkA. Cells were placed onto glass slides (Fisher, Loughborough, UK) at 5×10^4 per slide, using a cytospin centrifuge (Shandon, Southern Instruments Ltd, Runcorn, UK). Cells were fixed with 10% neutral-buffered formaldehyde (Bios Europe Limited, Skelmersdale, UK) for 20 minutes, and washed with PBS. Cells were blocked and permeabilized in 10% goat serum, 1% BSA, and 0.1% triton (all from Life Technologies) in PBS for 1 hour at room temperature before staining for the nuclear marker ΔNp63. Cells were blocked in 10% goat serum and 1% BSA for 1 hour at room temperature before staining for the surface marker TrkA. The cells then were incubated with primary antibodies against ΔNp63 (clone 6190; Biolegend, Cambridge, UK) and TrkA (clone 763; Santa Cruz Biotechnologies, Dallas, TX, USA) diluted 1:100 in PBS overnight at 4°C. After PBS washes, cells were incubated with a secondary antibody, Alexafluor 488 goat-anti-rabbit IgG (Life Technologies) 1:250 in PBS for 1 hour at room temperature. Further PBS washes were applied before and following counterstaining with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes, and the slides mounted with aqueous fluorescent mounting medium (Dako, Ely, UK) and imaged on a Nikon TiE fluorescent microscope

(Nikon, Tokyo, Japan). HeLa and MCF7 cells were used as positive controls for ΔNp63 and TrkA staining, respectively. Goat serum alone (5%) was used as a negative control. The percentage of cells expressing ΔNp63 or TrkA per eye was calculated over an average of 5 random fields of view in triplicate experiments yielding 15 images for each donor.

Preparation of Murine J23T3 Fibroblasts Feeder Layers

Murine J23T3 fibroblasts were cultured in high glucose (4.5g/L) DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FCS, 1% penicillin-streptomycin, and 1% fungizone until confluence. Before use as a feeder layer, the cells were inactivated by exposure to 10 µg/mL mitomycin C for 3 hours at 37°C. Cells then were harvested using 0.05% trypsin/0.02% EDTA and stored in liquid nitrogen with 10% dimethyl sulfoxide (DMSO) until use. Upon defrosting, cell viability was assessed by 0.4% trypan blue assay and cells plated as a feeder layer at 1×10^5 viable cells/cm².

CFE Assays

Cells were plated at 6×10^4 cell/cm² onto a preseeded inactivated J23T3 feeder layer on tissue culture wells (Greiner, Stonehouse, UK) and grown in limbal epithelial medium, which was refreshed every 2 to 3 days. After 14 days in culture, the cells were harvested using 0.05% trypsin/0.02% EDTA and CFE assays performed at passage 1. Viable limbal cells (as assessed with 0.4% trypan blue) were seeded at 2×10^3 , 3×10^3 , and 4×10^3 cells per 9.6 cm² well (Greiner) containing a preseeded inactivated J23T3 feeder layer. After 14 days, cells were fixed with methanol (Fisher Scientific, Loughborough, UK) at -20°C for 10 minutes and stained with 1% rhodamine B in methanol for 10 minutes at room temperature, washed with PBS, and air-dried. Colonies per well were counted and the CFE determined according to the number of colonies formed per number of cells seeded across 3 wells.

Analysis

The source of tissue (fresh versus stored) and donor factors (age, sex, death to retrieval time, retrieval to organ culture, and time in organ culture) were tested for significant associations with total cell number and/or cell death using a general linear multivariate model. Univariate comparisons between the sources of tissue were made using the Mann-Whitney *U* test or *t*-test (CFE). A Bonferroni correction was made for multiple tests.

RESULTS

Limbal tissue was obtained from 94 donors: 34 from fresh (mean age, 81.1 years; SD, 11.7) and 60 from stored (mean age, 63.2 years; SD, 15.15) tissue. Death to retrieval time was 24.1 hours (SD, 8.41) for fresh and 17 hours (SD, 5.52) for stored tissue. For stored tissue, mean time between retrieval and storage in organ culture media was 15.38 hours (SD, 6.28) and the mean time in organ culture was 33.37 days (SD, 7.52). For fresh tissue the average time between retrieval of the eyes and harvest of the limbal cells was 30.75 hours (SD, 30.87).

Cell Number and Viability

We used 34 fresh and 58 stored eyes to determine cell numbers, and 27 fresh and 49 stored eyes used to determine cell viability. The total number of cells obtained per eye from

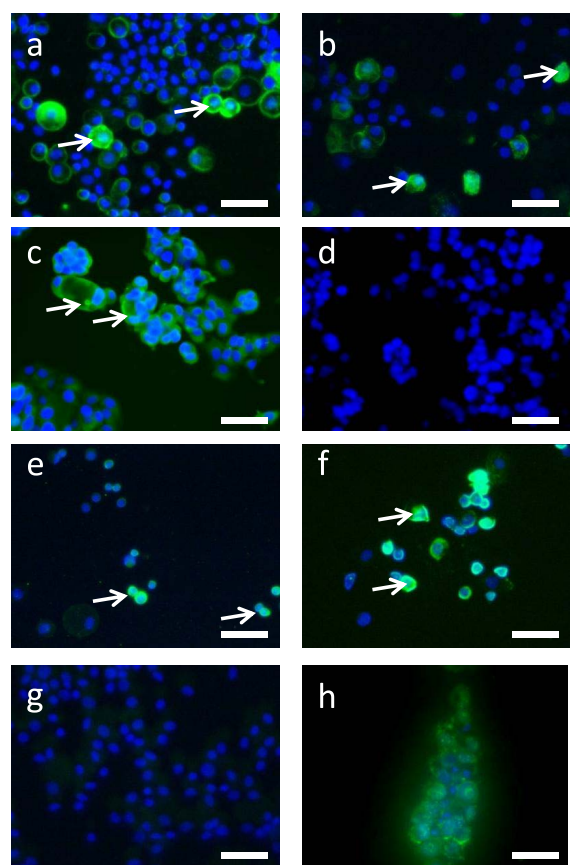
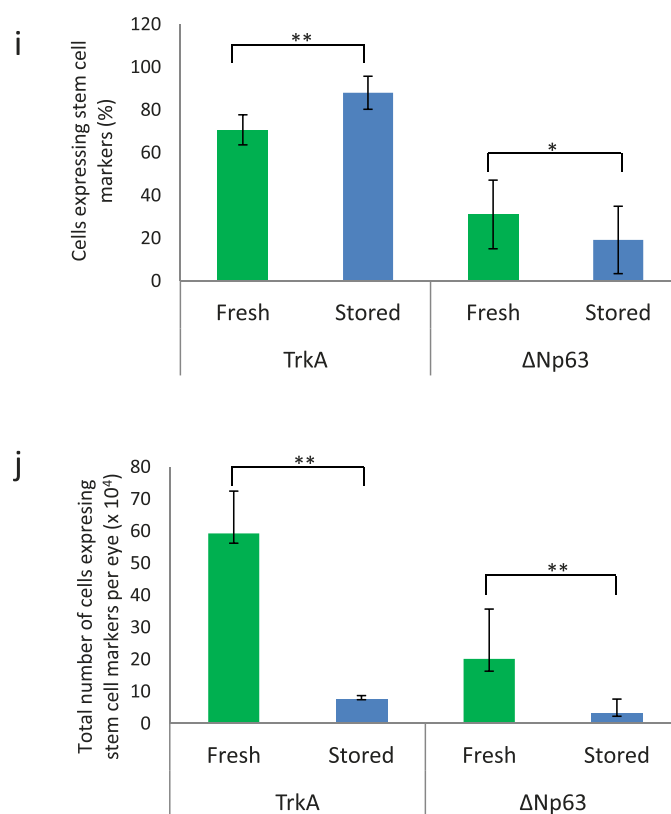


FIGURE 1. Photomicrographs of immunofluorescent staining for TrkA (arrows) in cells from (a) fresh and (b) stored tissue, with (c) MCF7-positive control and (d) -negative control. Photomicrographs of immunofluorescent staining for Δ Np63 (arrows) in cells from (e) fresh and (f) stored tissue, with (g) negative control and (h) positive control (HeLa cells). Scale bars: 50 μ m. Histograms demonstrating percentage (i) and total cell expression per eye (j) for TrkA and Δ Np63 (** $P < 0.01$, * $P \leq 0.05$). Error bars: ± 1 SEM.



fresh tissue (35.34×10^4 ; SD, 17.39) was three times greater than that obtained from stored tissue, (11.24×10^4 ; SD, 11.57; $P < 0.01$). In addition, there also was a significantly higher percentage of viable cells harvested from fresh (91.9%; SD, 5.7) compared to stored (85%; SD, 10.8; $P < 0.01$) tissue.

Although there were no significant associations for either fresh or stored tissue, between death to retrieval and total ($P = 0.16$) or nonviable ($P = 0.56$) cells, or between retrieval to organ culture and total ($P = 0.22$) or nonviable ($P = 0.56$) cells, the times were skewed to very long postmortem times with only 8 of all 94 donors having death to retrieval times of less than 12 hours.

Expression of Putative Stem Cell Markers

For fresh and stored tissue, 4 eyes (4 donors) were used to determine TrkA expression and 3 eyes (3 donors) were used to determine Δ Np63 expression. Expression of Δ Np63 and TrkA was demonstrated in all samples in cells from fresh and stored tissue with TrkA in greater numbers than Δ Np63 (Fig. 1). The total number of cells expressing either TrkA or Δ Np63 was higher, from fresh compared to stored tissue. The total number of cells per eye expressing TrkA was 59.24×10^4 (SD, 13.21×10^4) for fresh compared to 7.65×10^4 (SD, 1.05×10^4) for stored tissue ($P < 0.01$), and for Δ Np63 it was 20.19×10^4 (SD, 15.5×10^4) for fresh compared to 3.28×10^4 (SD, 4.33×10^4) for stored tissue ($P < 0.01$). That is, the number of cells per eye expressing either TrkA or Δ Np63 was 7.8 and 6.2 times higher, respectively, from fresh compared to stored tissue.

Colony-Forming Efficiency

Three eyes (3 donors) were used to calculate CFE for fresh and 4 eyes (4 donors) for stored eyes. The CFE of limbal cells from fresh tissue was significantly higher ($1.42\% \pm 0.12$) compared to that of stored tissue ($0.43\% \pm 0.15$; $P < 0.01$; Fig. 2).

Donor Age, Cell Numbers, and Viability

For fresh tissue, there were no significant associations between either the total numbers of cells ($P = 0.35$), proportion of nonviable cells ($P = 0.49$), or CFE ($P = 0.41$) with increasing donor age. In contrast, however, for stored tissue, there was a significant inverse relationship between donor age and the total number of cells isolated ($R^2 = 0.27$, $P < 0.01$; Fig. 3) together with a trend towards a lower proportion of viable cells ($P = 0.056$), but not with CFE ($P = 0.69$).

DISCUSSION

The treatment of limbal stem cell failure using ex vivo expanded or in situ transplants of limbal epithelial stem cell grafts has been an important therapeutic advancement. The challenge remains, however, on how to achieve long-term clinical success. A key factor determining this is likely to be the "stemness" of the transplanted cells,^{19,20} which, in turn, may be affected by storage of limbal tissue before cell harvesting. Limbal epithelial cultures for transplantation often are obtained from stored corneoscleral tissue.^{9,10,21} Previous studies have

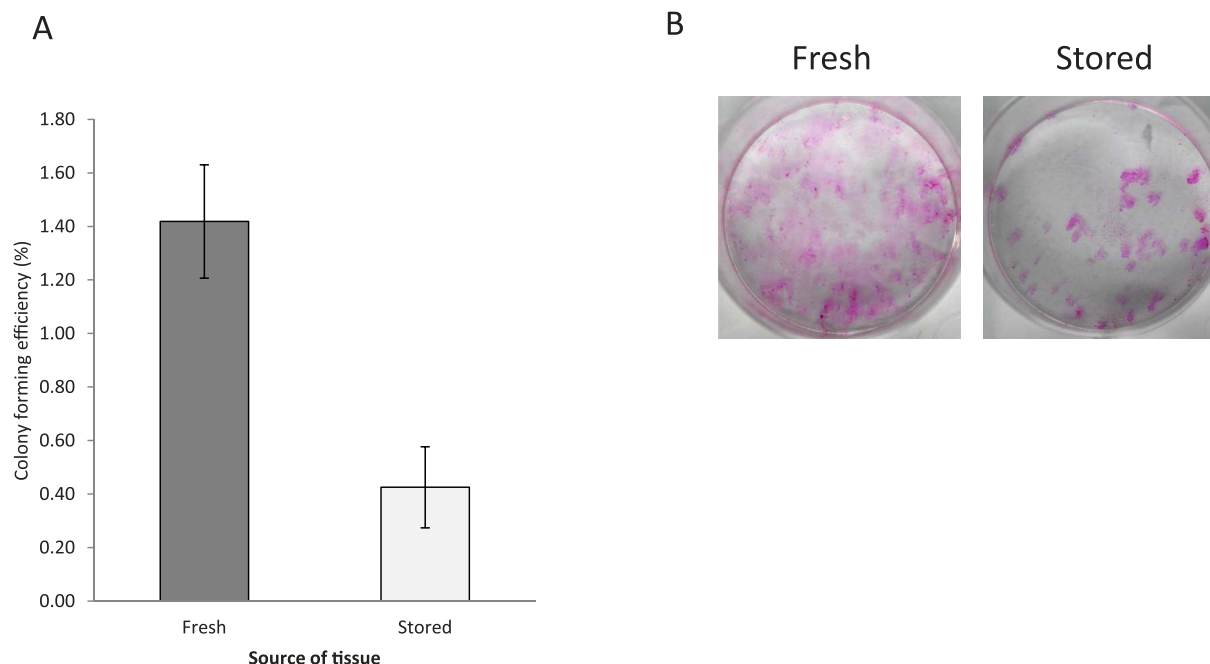


FIGURE 2. Colony-forming efficiency (CFE) for fresh and stored limbus. **(A)** Histogram demonstrating the mean CFE of limbal cells from fresh and stored tissue. Significantly higher CFE was demonstrated from cells isolated from fresh tissue ($*P < 0.01$). Error bars: ± 1 SEM. **(B)** Images of colonies stained with Rhodamine B.

investigated the effect of storage of corneoscleral discs on the proliferation of limbal cultures. While stored corneoscleral rings can produce successful cultures either by explant culture^{15,17,22} (Baylis OJ, et al. *IOVS* 2012;53:ARVO E-Abstract 3502) or single cell suspension,^{11,18} cultures from stored tissue grew more slowly¹⁵ and were less likely to achieve confluence¹⁷ compared to those from fresh tissue. Only two of the studies used fresh tissue as a comparison to stored tissue.^{15,17} Other studies have investigated associations between eye bank

parameters, donor age, and proliferation of explant outgrowths^{18,22} or effects of isolation techniques and culture conditions.¹¹ In these studies, an assessment of the type of cells present, that is, differentiated corneal or stem cell, was investigated qualitatively and only following cell culture to either confirm a mixed population of cells^{11,18,22} or to assess the number of cell layers.¹⁷

To our knowledge, no study has investigated the effect of organ storage on cell number, cell viability, and putative stem cell marker expression before culture, compared to that obtained from fresh tissue. This is important as cell culture may alter the characteristics of human limbal epithelial cells. Therefore, we sought to investigate how these parameters, including clonogenic ability, were affected by storage for up to 1 month in organ culture to characteristics human limbal stem cells before and following ex vivo culture.

Cell numbers, viability, and (quantitative) expression of stem cell markers were investigated immediately after harvesting the cells by trypsinization and before establishing culture. We found that the total number of cells obtained per eye was significantly higher from fresh than stored tissue. This would suggest that epithelial cells were mechanically debrided and/or sloughed away during storage; an entity that has been described previously during storage in either organ culture²³ or hypothermic media.¹² Indeed, the basal epithelial layer may be all that remains.²⁴ Since the stem cell niche resides within the basal layer of the limbal epithelium,²⁵ stem cells may be protected in part from this process. Although unknown, once the superficial terminally differentiated epithelial cells have been shed during storage, exposure of the stem cells may make them more susceptible to environmental change, such as in vitro storage, particularly because stem cells are very dependent on the specificity of their niche to survive and function properly.²⁶

A significantly lower proportion of viable cells were obtained from stored tissue. Given that the mean time in organ culture in this study was 24.77 days (range, 16–29 days) and that the majority of detrimental metabolic changes,

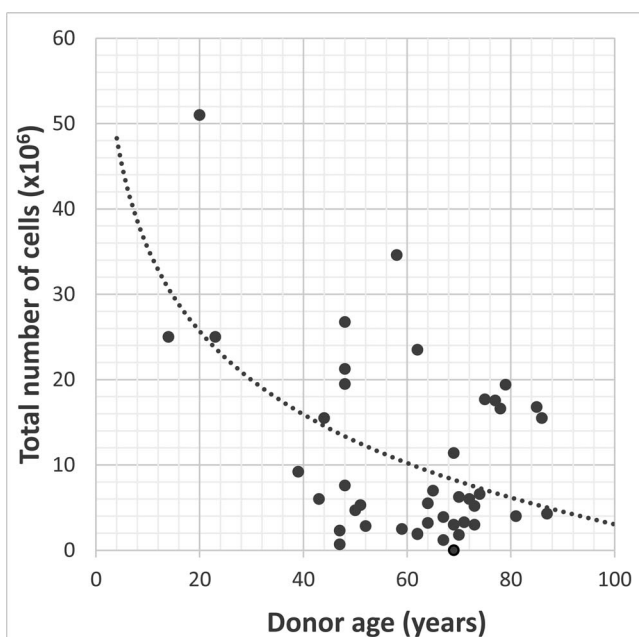


FIGURE 3. Scatter plot showing an inverse relationship between donor age and the total number of cells isolated from stored tissue ($R^2 = 0.27$, $P < 0.01$).

apoptosis, and cell death has been reported in the first 2 weeks of storage²⁷⁻²⁹ this is perhaps to be expected. Therefore, it is noteworthy that, despite longer postmortem retrieval times for fresh than stored eyes (24 and 17 hours, respectively), a higher percentage of viable cells were obtained from fresh tissue. This would indicate that the storage has a higher impact on the quality of the limbal epithelial stem cells than does time between donor death to retrieval. In line with most other studies,^{15,17,18,22} we found no correlation between time in organ culture and cell number or viability. The lowest storage time, however, was 16 days. Since studies indicate that most detrimental changes occur in the first 2 weeks of culture,²⁷⁻²⁹ investigating storage periods lower than this would be important.

Although we found no significant association between death to retrieval times and total cell number or number of viable cells the postmortem times for stored and fresh tissue were very long with only 8 of 94 donors having death to retrieval times of less than 12 hours. This may account for the differences in finding of other groups,^{18,22} which showed that higher death to retrieval times were associated with decreased cell growth in culture.

Both Δ Np63 and TrkA (the high affinity receptor for nerve growth factor) are more highly expressed in the basal layer of the limbal epithelium,^{30,31} and both costain with each other and are found in the ABCG2 side population of cells.^{31,32} Δ Np63 is essential to epithelial stem cell regeneration and is thought to identify cells in a proliferative state.³³ Therefore, both are considered putative limbal stem cell markers. Since previous studies have suggested that cells are lost via sloughing from the cell surface during organ culture²³ we investigated this by using the expressed putative stem cell markers Δ Np63 and TrkA to differentiate between basal and superficial cell layers. The total number of cells expressing Δ Np63 or TrkA per eye (taking into account the much lower overall cell numbers from the stored tissue) was 6.2 and 7.8 times greater, respectively, in fresh than in stored tissue. This would suggest that either there is a substantial loss of cells in the basal layer in addition to sloughing from the surface or that the stem cell properties are being lost due to the storage environment. Cultures from fresh tissue demonstrated significantly higher CFE than those from stored tissue, which supports the work of Komuro et al.,¹³ who demonstrated that apoptosis occurs in all cell layers during hypothermic storage.

The high yield of cells for the cytopsin experiments meant that there was not enough tissue to examine the capacity to form epithelial sheets in this study. Since it has been postulated that the number of stem cells within limbal tissue is directly associated with the probability of obtaining a successful culture or graft,^{19,20} the loss of stem cells during storage could indicate a poorer prognosis of tissue grafts if these cultures were used clinically. Moreover, due the paucity of cells remaining following clonogenic and cytopsin analysis, it was not possible to directly assess graft culture formation and integrity. While we have demonstrated that current organ culture methods of tissue preservation are associated with detrimental effects on limbal tissue, such as significantly reducing the total cell number, cell death, expression of stem cell markers, and CFE, there still may be advantages to using stored tissue. Storage offers logistical benefits, such as planned surgery and the exclusion of microbial infection, and for corneal transplants, a lower numbers of antigen-presenting cells.³⁴ In this study, we found that the yield of cell numbers from stored eyes was significantly greater from younger donors (Fig. 3). Other studies also have found that higher donor age was associated with lower cell proliferation of limbal ring cells in culture.^{15,18} This would indicate that if stored tissue were to be used, it should be from younger donors.

Corneal storage methods have seemingly been designed for the preservation of the corneal endothelium. It is possible that making some significant changes to this protocol might favor limbal epithelial stem cell survival. For example, during eye bank storage at 37°C, the medium typically is left unchanged for the whole banking period of up to a month. During this time, essential media components are depleted and waste products accumulate causing detrimental changes to the cells, such as intracellular oedema.¹² Given the majority of detrimental metabolic changes²⁸ and cell death²⁷ occurs early, it may be that much shorter storage periods, regular changes of media or different media would improve the survival of stem cells. Reducing the time between death and retrieval also may improve the condition of the epithelium. Thus, further research into alternative storage methods for limbal epithelium is warranted. Indeed, a recent study has shown that tissue storage in Optisol at 4°C with airlift culture results in a healthier epithelium with increased cell proliferation, higher expression of stem cell markers, and less cell death over a limited period.³⁵ If tissue is to be used for keratoplasty surgery and limbal stem cell transplantation therapies, a compromise in storage techniques may need to be sought.

Despite the limitations of the study, we provided good evidence that fresh tissue is more likely to provide a larger and more viable stem cell population than that obtained from organ-cultured tissue; which, in turn, should lead to an increase in the success of ex vivo expanded clinical therapies.

Acknowledgments

The authors thank the Specialist nurses at Tissue Services, NHS Blood and Transplant, UK for obtaining research consent for fresh tissue; the Manchester Eye Bank for receipt of stored tissue; Sajjad Ahmad, University of Liverpool for the gift of the J23T3 cell line; and NHS Blood and Transplant, UK and the Foundation for the Prevention of Blindness, Liverpool, UK for their generous funding. Supported by NHS Blood and Transplant, UK PG08-3; Foundation for the Prevention of Blindness, Liverpool, UK.

Disclosure: **S.L. Mason**, None; **R.M.K. Stewart**, None; **C.M. Sheridan**, None; **F. Keshtkar**, None; **P. Rooney**, None; **E. Austin**, None; **U. Schlötzer-Schrehardt**, None; **F.E. Kruse**, None; **S.B. Kaye**, None

References

- Ahmad S. Concise review: limbal stem cell deficiency, dysfunction, and distress. *Stem Cells Transl Med.* 2012;1: 110-115.
- Pellegrini G, Traverso CE, Franz AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1997; 349:990-993.
- Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ. Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. *Invest Ophthalmol Vis Sci.* 2000;41:2506-2513.
- Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S. 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. *J Cell Biochem.* 2011;112:993-1002.
- Croasdale CR, Schwartz GS, Mallin J, Holland EJ. Kerato-limbal allograft: recommendations for tissue procurement and preparation by eye banks, and standard surgical technique. *Cornea.* 1999;18:52-58.
- Brightbill FS, McDonnell PJ, McGhee CN, Farjo AA, Serdarevic O. *Corneal Surgery: Theory Technique and Tissue.* 4th ed. Amsterdam, The Netherlands: Elsevier Health Sciences. 2008.
- Armitage WJ. Preservation of human cornea. *Transfus Med Hemother.* 2011;38:143-147.

8. Grueterich M, Espana E, Tseng SCG. Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. *Invest Ophthalmol Vis Sci.* 2002;43:63-71.
9. Li W, Hayashida Y, He H, Kuo C, Tseng SC. The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane. *Invest Ophthalmol Vis Sci.* 2007;48:605-613.
10. Kolli S, Lako M, Figueiredo F, Mudhar H, Ahmad S. Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane. *Regen Med.* 2008;3:329-342.
11. Tovell VE, Massie I, Kureshi AK, Daniels JT. Functional limbal epithelial cells can be successfully isolated from organ culture rims following long-term storage. *Invest Ophthalmol Vis Sci.* 2015;56:3531-3540.
12. Means TL, Geroski DH, L'Hernault N, Grossniklaus HE, Kim T, Edelhauser HF. The corneal epithelium after optisol-GS storage. *Cornea.* 1996;15:599-605.
13. Komuro A, Hodge DO, Gores GJ, Bourne WM. Cell death during corneal storage at 4 C. *Invest Ophthalmol Vis Sci.* 1999;40:2827-2832.
14. Brzeszczynska J, Ramaesh K, Dhillon B, Ross JA. Molecular profile of organ culture-stored corneal epithelium: LGR5 is a potential new phenotypic marker of residual human corneal limbal epithelial stem cells. *Int J Mol Med.* 2012;29:871-876.
15. James SE, Rowe A, Ilari L, Daya S, Martin R. The potential for eye bank limbal rings to generate cultured corneal epithelial allografts. *Cornea.* 2001;20:488-494.
16. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. *Exp Eye Res.* 2004;79:41-49.
17. Vemuganti GK, Kashyap S, Sangwan VS, Singh S. Ex-vivo potential of cadaveric and fresh limbal tissues to regenerate cultured epithelium. *Indian J Ophthalmol.* 2004;52:113-120.
18. Zito-Abbad E, Borderie VM, Baudrimont M, et al. Corneal epithelial cultures generated from organ-cultured limbal tissue: Factors influencing epithelial cell growth. *Curr Eye Res.* 2006;31:391-399.
19. Pellegrini G, Rama P, Matuska S, et al. Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regen Med.* 2013;8:553-567.
20. Rama P, Matuska S, Paganoni G, Spinelli A, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Medicine.* 2010;363:147-155.
21. Grueterich M, Espana EM, Touhami A, Ti SE, Tseng SCG. Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency. *Ophthalmology.* 2002;109:1547-1552.
22. Shanmuganathan V, Rotchford A, Tullo A, Joseph A, Zambrano I, Dua H. Epithelial proliferative potential of organ cultured corneoscleral rims; implications for allo-limbal transplantation and eye banking. *Br J Ophthalmol.* 2006;90:55-58.
23. Van Meter WS, Katz DG, White H, Gayheart R. Effect of death-to-preservation time on donor corneal epithelium. *Trans Am Ophthalmol Soc.* 2005;103:209-222.
24. Greenbaum A, Hasany S, Rootman D. Optisol vs dexsol as storage media for preservation of human corneal epithelium. *Eye.* 2004;18:519-524.
25. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature.* 1971;229:560-561.
26. Scadden DT. The stem-cell niche as an entity of action. *Nature.* 2006;441:1075-1079.
27. Pels E, Schuchard Y. Organ-culture preservation of human corneas. *Doc Ophthalmol.* 1983;56:147-153.
28. Redbrake C, Salla S, Frantz A, Reim M. Metabolic changes of the human donor cornea during organ-culture. *Acta Ophthalmol Scand.* 1999;77:266-272.
29. Crewe JM, Armitage WJ. Integrity of epithelium and endothelium in organ-cultured human corneas. *Invest Ophthalmol Vis Sci.* 2001;42:1757-1761.
30. Qi H, Chuang EY, Yoon KC, et al. Patterned expression of neurotrophic factors and receptors in human limbal and corneal regions. *Mol Vis.* 2007;13:1934-1941.
31. Qi H, Li D, Shine HD, et al. Nerve growth factor and its receptor TrkA serve as potential markers for human corneal epithelial progenitor cells. *Exp Eye Res.* 2008;86:34-40.
32. Epstein SP, Wolosin JM, Asbell PA. P63 expression levels in side population and low light scattering ocular surface epithelial cells. *Trans Am Ophthalmol Soc.* 2005;103:187-199; discussion 199.
33. Senoo M, Pinto F, Crum CP, McKeon F. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell.* 2007;129:523-536.
34. Armitage W. The effects of storage of corneal tissue on langerhans cells. *Eye (Lond).* 1995;9:228-232.
35. Li C, Dong N, Wu H, et al. A novel method for preservation of human corneal limbal tissue. *Invest Ophthalmol Vis Sci.* 2013;54:4041-4047.